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DIMORPHISM OF *PITYROSPORUM ORBICULARE* IN A DEFINED CULTURE MEDIUM

MICHAEL DORN, M.D., AND KLAUS ROEHNERT

Department of Dermatology, University of Munich, Munich, West Germany

Dimorphism of *Pityrosporum orbiculare* was induced in an artificial culture medium which consisted of 0.05 M glycine in 0.03–0.06 M ammonium phosphate buffer (pH 5.6), salts, glucose, and Tween-80. Of the strains tested, 78 to 85% produced hyphal elements from up to 8.5% of yeast cells. Morphologic features of *P. orbiculare* in culture were examined by scanning electron microscopy and compared with the appearance of fungus from clinical lesions of tinea versicolor. Morphologically, the fungi obtained in vivo and in vitro were identical.

Pityrosporum orbiculare is the causative agent of the superficial fungal infection tinea versicolor [1]. This lipid-dependent yeast can be regularly isolated from clinical lesions [2]. Furthermore, there are experiments in humans pointing out its etiologic significance [3]. Morphologic investigations and immunofluorescence have shown the striking similarity of the cultured yeast and tissue phases of the fungus [4–6].

P. orbiculare is thought to be a dimorphic fungus capable of yeast–mycelial conversion. The simultaneous occurrence of characteristic hyphal elements and spheroidal budding yeast cells is typical for the parasitic stage as seen in tinea versicolor. As part of the normal skin flora and in cultures, the fungus exhibits mainly the yeast phase, which is considered to be a saprophytic stage [3,7].

The in vitro induction of the yeast–mycelial conversion has been unsuccessful so far and there is therefore a missing link [1,3,8,9]. We wish to report a culture medium which allows the development of hyphal elements of *P. orbiculare* in a chemically defined environment.

MATERIALS AND METHODS

Fungi

Twenty-two stock cultures of *P. orbiculare* were employed, all of which conformed to the biochemical and morphologic criteria for their species [1]. The strains were originally isolated from scales of tinea versicolor and held in continuous subcultures (2 to 8 months) by alternating solid and liquid media [10,11]. Strains that had not lost their characteristic morphology were selected. None of these strains developed hyphal elements when maintained on routine media. For comparison, control studies were done with *P. ovale*, which were obtained from CBS (Centraalbureau Voor Schimmelcultures, Baarn, Netherland) as strains No. 1070, No. 1078, No. 4162. Strains of *P. orbiculare* are not available at the present time from CBS.

Media

Solid medium for routine cultivation and maintenance of strains contained 2% olive oil, 0.2% Tween-80 (polyoxyethylene sorbitan monooleate), 1% phytone (BBL, Cockeysville, Md.), 0.1% yeast extract (Difco Laboratories, Detroit, Mich.), 2% glucose and 1.5% bacto-agar (Difco). Antibiotics (chloramphenicol 50 mg/l and cycloheximide 500 mg/l) were added prior to autoclaving. The pH was adjusted to 5.6 with 0.1 N HCl [10].

Liquid medium for continuous subculture contained 7.5% Tween-80, 0.25% yeast extract (Difco), 3.5% dehydrated Czapek–Dox broth (Difco) in distilled water with addition of chloramphenicol and cycloheximide, pH 5.6 [11].

Filamentation medium consisted of 0.06 M $(NH_4)_2PO_4$–$(NH_4)HPO_4$ buffer, pH 5.6, with 1.0 gm KNO_3, 0.13 gm $MgSO_4 \cdot 7\,H_2O$, 0.007 gm $FeSO_4$, 1.3 gm NaCl, 13.0 gm glucose, 50 ml Tween-80, 0.05 gm chloramphenicol, and 0.5 gm cycloheximide per liter. To the autoclaved medium, glycine dissolved in distilled water and filtered sterile was added to reach a final concentration of 0.05 M. The same medium with 4% Tween-80 and 2% bacto-agar (Difco) was used for plates. For pilot studies the glycine concentration was varied from 0.01 M to 0.25 M in 0.1 M buffered medium. Additionally, the molarity of the buffer (0.1 M, 0.06 M, 0.03 M ammonium phosphate) and the concentration of constituents except the antibiotics were changed.

All reagents, if not otherwise indicated, were obtained from Merck (Darmstadt, Germany) and were of analytical grade.

Experimental Conditions

Three strains of *P. orbiculare* and one strain of *P. ovale* (CBS 1070) were harvested after 7 days of growth in liquid medium, repeatedly washed in sterile distilled water and adjusted to an optical density of 20% transmission at a wavelength of 550 nm (spectralphotometer 340, Bausch & Lomb, Rochester, N.Y.). One hundred-milliliter sealed flasks containing 50 ml of liquid filamentation medium (0.1 M) were inoculated with 1 ml of yeast suspension. Cultures were held at 29°C, being shaken at 110 gyrations per min. After 7 days of incubation, cultures were examined for formation of hyphae,

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Reprint requests to: Dr. M. Dorn, Department of Dermatology, University of Munich, Frauenlobstrasse 9, 8000 Munich 2, West Germany.

the cell yield was measured in a Coulter Counter (Coulter, Dunstdable, England) and the cells were compared to harvests from routine liquid media. The effect of various glycine concentrations was investigated under identical conditions.

Fifty-milliliter aliquots of liquid filamentation medium in 100-ml sealed flasks were inoculated with standardized amounts of *P. orbiculare* (14 strains) and *P. ovale* (3 strains). Seven-day-old cultures incubated at 29°C were harvested, washed 3 times with distilled water, and brought to an optical density of 10% transmission at 550 nm. Yeast cells and hyphae were counted in a hemocytometer and the percentage of filamentation determined (standard reference: 10,000 yeast cells). Under identical conditions the effects of buffer molarity and salt concentrations were determined. Twenty-two strains were tested in 0.1 M buffered medium, and 14 strains in both 0.06 M and 0.03 M buffered medium. Parallel cultures were run on solid filamentation media at 37°C and microscopically checked every second day for hyphae.

Scanning Electron Microscopy (SEM)

Seven-day-old cultures in liquid filamentation medium were harvested and washed 3 times with saline. Yeast suspensions were fixed in buffered formalin, pH 7.2, and dehydrated slowly with increasing concentrations of ethanol. Yeast suspensions in 100% ethanol were dropped directly on specimen holders, air dried, and coated with gold under a vacuum of 10^{-5} torr. The preparations were examined with a Cambridge Stereoscan Mk II a. For comparison unfixed preparations of tinea versicolor were examined. Microscopically proved lesions were directly stripped with a specimen holder by means of a cyanoacrylate adhesive (I.S. 12, Loctite, Dublin, Ireland).

RESULTS

Cultural Studies

Growth of *P. orbiculare* in filamentation media was suppressed compared to routine media. The harvest of yeast cells in liquid filamentation medium was only 20 to 30% compared to cultures in routine Czapek-Dox medium. Similarly, there was little growth on the filamentation plates. Solid filamentation medium was advantageous as it is clear and translucent and cultures can therefore be easily observed or photographed directly from the plates (Fig. 1). Filamentation agar was not suitable for the primary isolation of *P. orbiculare* from scales of tinea versicolor. Subcultures of *P. orbiculare* revealed germinating yeast cells and hyphae on these plates. Only strains were examined that had been capable of developing hyphae in liquid filamentation medium.

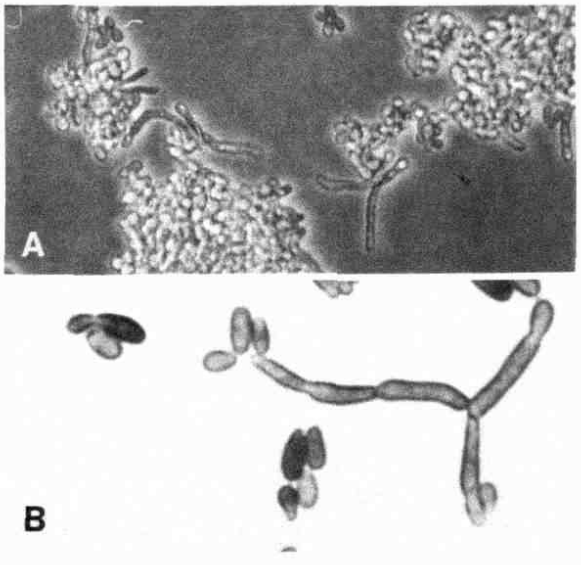

FIG. 1. *P. orbiculare* on solid filamentation medium. *A:* Phase contrast of agar plate (× 1,700). *B:* Smear stained with PAS (× 4,400).

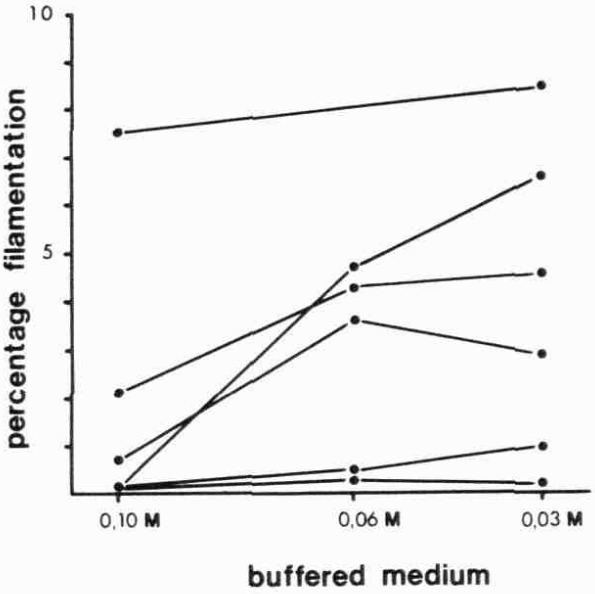

FIG. 2. *P. orbiculare* in liquid filamentation medium. Effects of various molarities of medium buffer on filament formation in 6 selected strains. Means of duplicate assays.

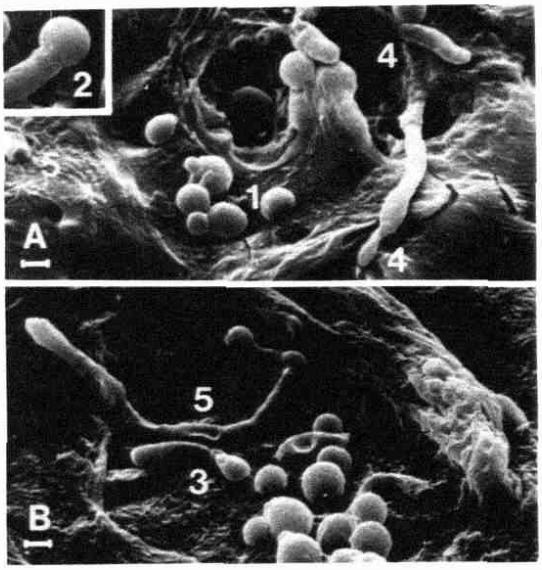

FIG. 3. Tinea versicolor. *A:* Filamentation of hyphae *(4). Inset:* Hyphal formation from a yeastlike phialide *(2). B:* Spore extrusion from hyphae *(3).* Cyanoacrylate strip (× 9,250). Scale bar indicates 2 μ in this and subsequent Figures. For *1–5* in Figures 3–6 see *Results*.

None of the three tested strains of *P. ovale* produced hyphae. The morphologic appearance of this species changed, however, to somewhat ovoid and relatively small cells. In this phase morphologic differentiation from *P. orbiculare* was difficult.

All 3 strains of *P. orbiculare* from the pilot studies were capable of hyphal formation in various degrees. After 7 days of growth in 0.1 M buffered medium, the addition of 0.05 M glycine resulted in 0.25%, 2.9%, and 2.85% hyphal formation. When glycine was lowered to 0.01 M, no hyphae developed despite undistributed growth of the yeast phase. Increase in glycine (0.25 M) resulted in no further stimulation of filament formation. In the pilot studies hyphal formation did not depend on whether the original inoculum was derived from liquid or solid routine cultivation media.

Results of various molarities of medium buffer in constant glycine molarity (0.05 M) are presented in Figure 2. Variability of strains in filament formation and higher individual rates of conversion with increasing dilutions are shown in 6 strains, which produced hyphal elements under any of the varied conditions. Individual ability to convert could be increased markedly by a factor of 1.2 to 330, the median being fivefold. Strains with good hyphal formation could be stimulated only to a lesser degree (Fig. 2). Thirteen of 22 strains developed hyphae in 0.1 M buffered medium; yield of hyphae varied from 0.02 to 7.4%. In diluted media 8 strains with proved ability to convert were tested, and 6 strains which had not shown yeast-mycelial conversion in 0.1 M buffered medium were tested. The remaining 8 strains were not further cultured. With 0.06 M buffered medium 11 of these 14 strains developed hyphae and in 0.03 M buffered medium 12 of 14. The latter included 3 strains which developed no hyphae in 0.1 M buffered medium, and 1 strain which was negative in 0.06 M medium. In general, dilution of the buffered medium led to improved selection of hyphae-forming strains, and the rate of hyphal formation was increased. There were 2 strains of *P. orbiculare* which, like control strains of *P. ovale*, did not show yeast-mycelial conversion under any of the varied conditions.

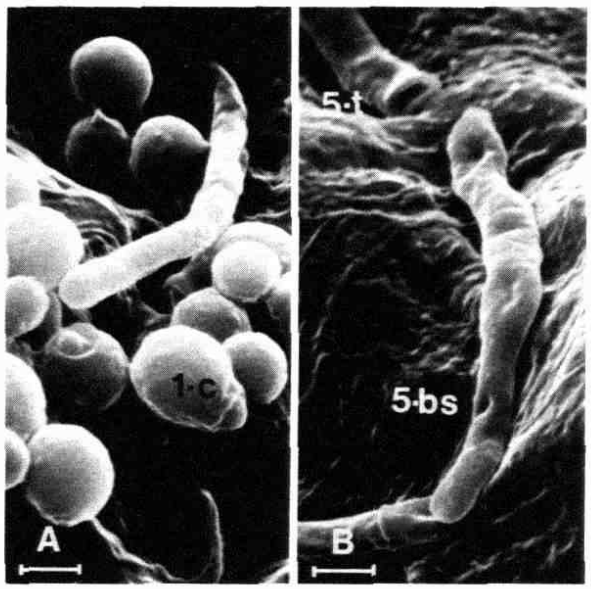

FIG. 4. Tinea versicolor. *A:* Budding yeast cells showing collarettes *(1c)*. *B:* Bud scar *(5bs)* and tubelike hole *(5t)* in hyphae. Cyanoacrylate strip (× 18,700).

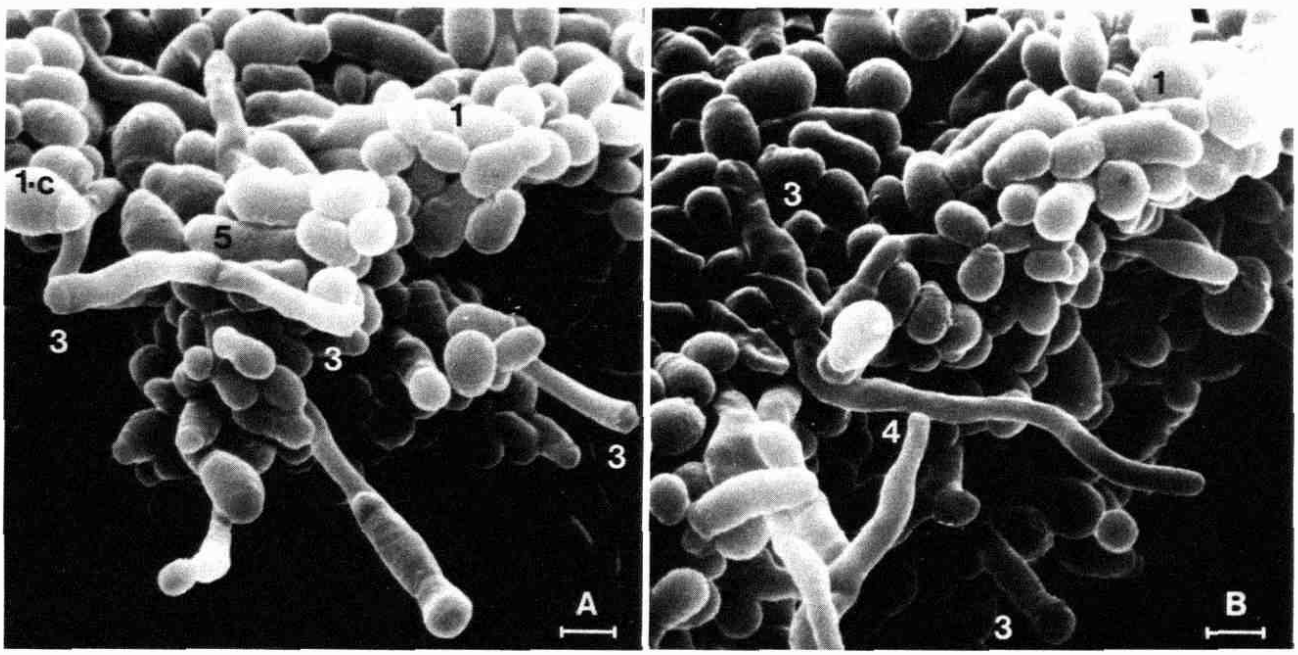

FIG. 5. *P. orbiculare* from liquid filamentation medium. *A:* Spore extrusion from hyphae *(3)* and septation of hyphae; collarette formation in budding yeasts *(1c)*. *B:* Simultaneous budding and filamentation from hyphae *(3)* (× 17,000).

Morphologic Characteristics

Representative natural-occurring forms of the fungus within clinical lesions of tinea versicolor are shown in Figures 3 and 4. Appearance of *P. orbiculare* in liquid filamentation medium can be seen in Figures 5 and 6. Hyphal phases of *P. orbiculare* are similar under in vitro and in vivo conditions; minor variations are attributable to different stages of filamentation and sporulation. The yeast forms from the culture display a greater variety than is found from scales of clinical lesions. Cells vary considerably in size and shape, being mostly oval or elongated. This is due to higher numbers of germinating and budding cells.

Reproduction and growth in *P. orbiculare* takes place in five ways, which are identical in vivo and in vitro. In the following description 1–5 are simultaneously used within the text and the Figures for better identification.

1. The main characteristics of this species are round, ovoid, or bottle-shaped yeast cells (phialides), which form single, apical, spherical to oval buds (Figs. 3–5). During extrusion of these buds the free rim of the cell wall of the parent cell forms a clearly visible collarette (Figs. 4*A*, 5*A*). After fission of daughter cells bud scars may remain.

2. In the same manner of extrusion, hyphae may develop from yeastlike phialides. In Figures 3 (*inset*) and 6*A* this stage of yeast–mycelial transition is demonstrated. The collarette signifies the parent cell.

3. Hyphae can also extrude spores at their free ends, again with a formation of a collarette (Figs. 3*B*, 5). As a rule, spores are smaller than blastospores of yeast cells. It can be assumed that such spores form also at the end of hyphae like an *anhynum* (Fig. 6*A*).

4. Filament formation is also possible in hyphae. In this case narrow, pointed, curved hyphae are visible in early stages (Figs. 3*A*, 5*B*). This phenomenon may be *repetitive*, so that some hyphae attain a telescopic appearance. Under our experimental culture conditions there was frequently simultaneous sporulation on one side and filament formation on the other side within the same hypha (Fig. 5*B*).

5. Marginal budding in hyphae may occur (Fig. 6*C*). We have not observed this mechanism under in vivo conditions. Rarely, however, bud scars were seen alongside hyphae (Fig. 4*B*). Similar lateral filamentation of hyphae seems possible as indicated by branching mycelia (Fig. 6*B*). Not infrequently, well-septated hyphae break apart exposing tubelike cavities, similar to those seen after extrusion of spores from hyphae (Figs. 3*A*, 5*B*, 6*A*).

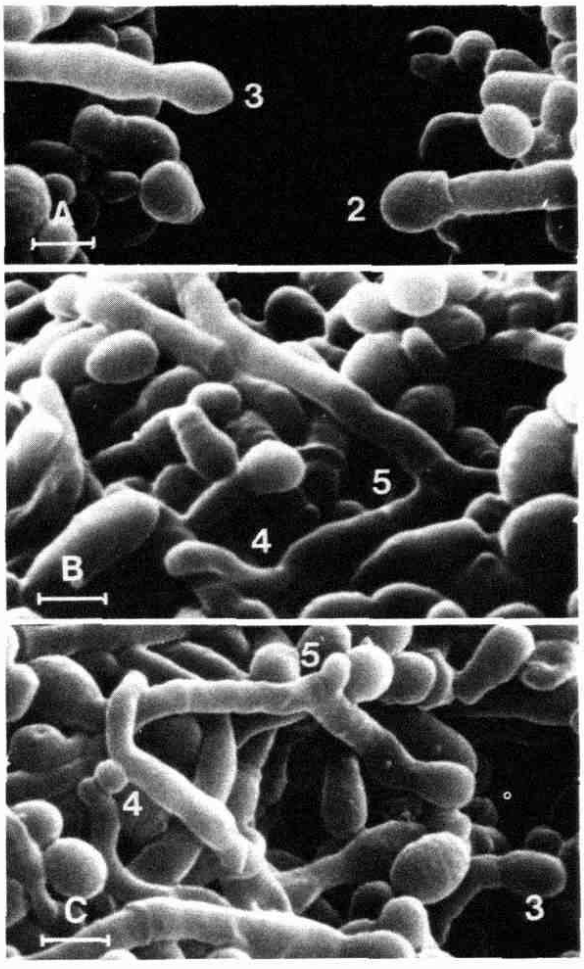

FIG. 6. *P. orbiculare* from liquid filamentation medium. *A:* Hyphal formation from yeast *(2)* and spore extrusion from hypha *(3)* (× 18,700). *B:* Branching mycelium *(5)* (× 21,700). *C:* Lateral budding from hyphae *(5)* (× 20,400).

DISCUSSION

There are only sparse reports in the literature of the occurrence of germinating tubes and hyphal elements in cultivated *P. orbiculare* [1–5, 9]. As a rule these are chance observations which were only possible on a few strains. Furthermore, these morphologic elements were mostly seen in colonies on first isolation but not in subsequent transfers [9]. Precise nutritional requirements for conversion of the yeast to the mycelial phase have not been established to our knowledge. The media described in this report allow with relative certainty the induction of dimorphism in *P. orbiculare*. Glycine appears to be the most important component, variations of other constituents have less influence; some type of starvation medium is required.

Marked differences among various strains exist for induction of hyphae. Two of our strains did not produce hyphae, although by morphologic criteria they were identified as *P. orbiculare*. On the other hand, none of the tested strains of *P. ovale* produced hyphae. This may be a way to differentiate between the two species which sometimes cannot be separated on morphologic terms [1,9].

Following earlier detailed light microscopic descriptions [4,12] of *Malassezia furfur*, the most informative knowledge of this fungus within lesions of tinea versicolor comes from SEM studies [13–16]. All the already described naturally occur-

ring stages of yeast–mycelial transition have been obtained under culture conditions in this investigation. A constant finding was hyphae which extruded phialospores on one end while forming filaments at the same time on the other end. This was much less evident in vivo. Earlier investigators speculated that this phenomenon took place only in the deeper layer of the stratum corneum [13], but we believe this stage of *P. orbiculare* occurs regularly under experimental conditions.

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